EXHIBIT D

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Original Contribution

A RAPID GAS CHROMATOGRAPHIC ASSAY FOR DETERMINING OXYRADICAL SCAVENGING CAPACITY OF ANTIOXIDANTS AND BIOLOGICAL FLUIDS

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Abstract—Herein, we report a new, rapid,and reliable method for measuring the protective antioxidant potential of pure antioxidant solutions or biological tissues. Peroxyl radicals generated by thermal homolysis of 2,2′-azobis-amidinopropane (ABAP) cause the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene; ethylene formation is monitored by gas chromatographic analysis of head space from the reaction vessel. The partial inhibition of ethylene formation in the presence of antioxidants that compete with KMBA for oxyradicals is the basis of the Total Oxyradical Scavenging Capacity Assay (TOSCA). The assay is shown to be reliable for quantifying ROS scavenging potential. The quantifiable parameters are consistent with the relative order of those predicted by the fluorescence- and oxygen electrode-based assays reported in the literature. Antioxidants competing for peroxyl radicals influenced the rate of KMBA oxidation in different ways, but the calculation of TOSC was not affected by such variations. Responses were linear over a wide range of sample concentrations and the TOSC values of classical soluble antioxidants showed the following relative order: Trolox > uric acid > ascorbic acid > GSH. The KMBA method was reliable for biological tissues; the TOSC for 1 μg rat liver cytosolic protein was 0.40 ± 0.02 and for the microsomal membrane, 0.15 ± 0.03. Soluble antioxidants accounted for 77% of the protective antioxidant potential in rat liver cytosol. When incorporated into the microsomal membrane, α-tocopherol markedly enhances antioxidant protection against peroxyl radical; thus, the assay is suitable for the assessment of fat-soluble antioxidants. © 1998 Elsevier Science Inc.

Keywords—Free radicals, Oxyradicals, Free radical scavengers, Antioxidant, ABAP, Peroxyl radicals

INTRODUCTION

Formation of reactive oxygen species (ROS) in aerobic organisms is an unavoidable consequence of the coupling of oxidative phosphorylation of ADP with the reduction of molecular oxygen by four electrons to water. Other well-recognized sources of ROS production include microsomal and photosynthetic electron transport chains, active phagocytosis, and the activity of several enzymes, for example, xanthine oxidase, tryptophan dioxygenase, diamine oxidase, prostaglandin synthase, guanyl cyclase, and glucose oxidase, which produce different ROS as intermediates.[1–9]

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Xenobiotics and environmental pollutants may increase the intracellular formation of ROS, for example, through the Fenton reaction involving trace metals such as iron and copper[10,11] or redox cycling of several classes of organic compounds.[12,13] During redox cycling certain molecules may be reduced to their corresponding free radical, which rapidly donates its free electron to molecular oxygen thereby producing the superoxide anion radical ($O_2^{\cdot -}$) and regenerating the parent compound to undergo another cycle.[12,13]

To counteract the biological potential of ROS formation cells have evolved complex antioxidant defenses of both specially adapted enzymes (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and smaller molecules such as vitamin E and β-carotene (as free radical scavengers in membranes), ascorbic acid, uric acid, and glutathione (for the aqueous

phase). In this respect, the intracellular production of ROS does not necessarily imply cellular toxicity, but oxidative stress will occur when ROS formation exceeds antioxidant defense capability. Oxidative stress has been linked to several cellular toxicity processes including damage to proteins, membrane lipid peroxidation, DNA alteration, enzyme inactivation,[14-16] and various pathologies including chemical carcinogenesis, heart disease, reperfusion injuries, rheumatoid arthritis, inflammation, and aging.[17-19]

Studies on oxidative stress have classically been approached by analysis of specific, single antioxidants, their modes of action, and their responses to different stressors. Variations in the levels or activity of antioxidant defenses have often been used to indicate ROS-mediated toxicity.[20-22] Although this approach has been useful in understanding specific relationships between various kinds of stressors and antioxidants, it is difficult from such data to evaluate the actual biological resistance to oxidative stress in quantifiable terms. For example, reduced levels of a putative protective agent may not result in oxidative damage in a tissue if compensated for by the presence of other defenses. It has been suggested that antioxidants may act cooperatively, thereby providing organisms with greater protection against ROS toxicity than expected by contributions of single antioxidants acting alone.[23]

A measure of the total absorbance capacity of oxyradicals by a tissue would provide better understanding of its resistance to toxicity caused by ROS, and some methods have been proposed for this purpose. Wayner et al.[24] measured the length of time ("time of induction") required to obtain maximum oxygen consumption in a system containing plasma peroxidizable material, a free radical generator, and plasma or specific antioxidants. By relating the results to the time of induction obtained with a known amount of the water-soluble vitamin E analogue, Trolox, a quantitative measure of the total radical-trapping antioxidant parameter (TRAP) could be defined.[23-25]

Glazer[26] described a method to assess the chemical damage caused by peroxyl and hydroxyl radicals to the protein phycoerythrin (PE) by measuring the decrease in its emission fluorescence as PE was oxidized. By measuring the inhibition of this fluorescence loss in the presence of different molecules, a method was proposed as a rapid screen for evaluation of free radical scavengers.[26,27] Largely based on this approach of Glazer, Cao et al.[28] and Ghiselli et al.[29] proposed two different modifications to quantify antioxidant behavior. The kinetics of PE fluorescence decay caused by peroxyl radicals is linear, but in the presence of an antioxidant there is a period of protection followed by a rapid loss of fluorescence. Cao et al.[28] calculated the net protection as the difference between the area under the kinetic curve of the sample and that of the control (net protection area), assigning 1 ORAC (Oxygen Radical Absorbance Capacity) unit to the net protection area obtained with 1 μM Trolox. Ghiselli et al.[29] measured the lag-phase, i.e., the time of complete protection of PE provided by plasma antioxidants. Pryor et al.[30] have reported a method for determining "antioxidant efficiencies" of purified molecules based on the rate of linoleic acid oxidation to its conjugated diene hydroperoxide in aqueous micelles, which is more sensitive than the oxygen uptake methods described above. Although quite capable of assessing antioxidant efficiencies of the various chemicals used, these authors did not study the effects of biological tissues, which undoubtedly would interfere with the UV detection of conjugated dienes in the micelles. Recently, Chevion et al.[31] proposed cyclic voltammetry as a different method to discriminate specific low molecular weight antioxidants in plasma (indicated by oxidation potential, $E_{1/2}$) as well as their concentrations (proportional to current heights, Ia).

In the present article, we describe a new, simple and reliable approach based on the reaction between peroxyl radicals (or hydroxyl or alkoxyl radicals) and α-keto-γ-methiolbutyric acid (KMBA), which is oxidized to ethylene[32] upon reaction with various ROS (equation 1).

$$\begin{aligned} &CH_3S{-}CH_2{-}CH_2{-}CO{-}COOH + {}^{\bullet}OOH(R) \\ &\quad \text{or } {}^{\bullet}OH(R) \rightarrow \tfrac{1}{2}(CH_3S)2 + (R)HOO^- \\ &\quad \text{or } (R)HO^- + CO_2 + CH_2{=}CH_2\uparrow \end{aligned} \quad (1)$$

Since first shown that ethylene is produced from methional by Pryor and Tang,[33] numerous investigators have used this or KMBA oxidation as an end point for the measure of reactive oxygen species production by biological systems. Ethylene is easily measured by gas chromatographic analysis of aliquots removed from the head-space of the reaction vessel. Herein, our purpose is not to measure ROS production per se by the KMBA reaction; rather, we describe an adaptation of this reaction for use as a quantifiable measure of the ability of biological tissues and various compounds or mixtures to scavenge potent ROS. We discuss the versatility of this method in relation to the unique property of KMBA to react with the numerous oxidants that can oxidize KMBA to ethylene, for example, alkoxyl radical,[32] HOCl,[34] and peroxynitrite.[35] The partial inhibition of ethylene production is the basis of the Total Oxyradical Scavenging Capacity (TOSC) assay. Ethylene formation in the control reaction does not reach completion during the assay, which obviates against

some of the problems reported by other investigators,[23-29] yet permits facile quantification of the oxyradical scavenging capacity of different samples. The gas chromatographic procedure described herein represents a simple alternative to the fluorometric and polarographic methods that have been developed. Such an alternative extends the capability of assessing absorbance capacity of antioxidants to many investigators who may not have ready access to the other technologies. Analytical details of the procedure are described and validation of the method is provided with different antioxidant solutions and biological fluids.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, bovine serum albumin (BSA), butylated hydroxyanisole (BHA), α-keto-γ-methiolbutyric acid (KMBA), melatonin, desferroxamine, reduced glutathione (GSH), oxidized glutathione (GSSG), and uric acid were purchased from Sigma Chemical Co. (St. Louis, MO); 2,2′-azobis-amidinopropane (ABAP) was obtained from Wako Chemicals (Richmond, VA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was from Aldrich Chemical Co. (Milwaukee, MI); DL-α-tocopherol acetate (vitamin E acetate) and β-carotene were purchased from ICN biomedicals, Inc. (Aurora, OH).

TOSC assay

Peroxyl radicals were generated by the thermal homolysis of ABAP at 35 and 39°C. Preliminary experiments (see Results) indicated as appropriate assay conditions 0.2 mM KMBA and 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4. The TOSC values were measured for different soluble antioxidants (GSH, ascorbic acid, uric acid, Trolox, and BHA) and for oxidized glutathione, BSA, and biological fluids. Although the rate of radical generation is approximately twofold greater at 39 than at 35°C, the TOSC values are not effected. Some combinations were also tested to detect possible synergistic interactions.

Reactions were carried out in 10 ml rubber septa-sealed vials in a final volume of 1 ml. The reactions were initiated by injection of 100 μl of 200 mM ABAP in water directly through the rubber septum. Ethylene production was measured by gas-chromatographic analysis of 1 ml aliquots taken directly from the head space of the reaction vials. By staggering the starting times for each vial, 8–10 serial samples can be monitored in sequence at 12-min intervals. Analyses were performed with a Hach-Carle (Series 100 AGC) gas chromatograph equipped with a 6-foot Poropack N column (Supelco) and a flame ionization detector (FID). The oven, injection and FID temperatures were respectively, 60°, 280°, and 190°C. Helium was used as the carrier gas at a flow rate of 30 ml/min.

Quantification of total oxyradical scavenging capacity (TOSC)

The area under the kinetic curve was mathematically calculated from the integral of the equation that best defines the experimental points for both the control and sample reactions. TOSC is then quantified according to equation 2, where ∫SA and ∫CA are the integrated areas from the curve defining the sample and control reactions, respectively.

$$\text{TOSC} = 100 - \left(\int \text{SA} / \int \text{CA} \times 100\right) \qquad (2)$$

Thus, a sample that displays no oxyradical scavenging capacity would give an area equal to the control ($\int SA/\int CA = 1$) and a resulting TOSC = 0. On the other hand, as $\int SA \rightarrow 0$ the hypothetical TOSC value approaches 100. Because the area obtained with the sample is related to that of the control, the obtained TOSC values are not affected by small variations in instrument sensitivity, reagents, or other assay conditions. The specific Total Oxyradical Scavenging Capacity was calculated by dividing the experimental TOSC by the molar concentration of the antioxidant or by the amount of protein in g used in the assay.

Tissue preparation

Rat livers were homogenized in 4 vol of STE (0.25 M Sucrose, 10 mM TRIS-HCl, 1 mM EDTA) buffer. This homogenization buffer had no effect on the TOSC assay. After initial centrifugation at 26,000 × *g* (4°C) for 20 min, the supernatants were further centrifuged at 105,000 × *g* (4°C) for 1 h. Cytosols were decanted and aliquoted for storage at −80°C. The resulting pellet from this centrifugation was washed once in 0.25 M KCl and centrifuged again at 105,000 × *g* for 1 h. The resulting pellet is the microsomal fraction, which was suspended in 0.25 M KCl 15% and stored at −80°C until used. Separation of the soluble and protein fractions of rat liver cytosol was performed by precipitation with 80% saturated ammonium sulfate[27] or by use of microconcentrators with a membrane cutoff of 3 kDa (Microcon 3, Amicon Inc., Beverly, MA). The $(NH_4)_2SO_4$-precipitated protein was washed three times and redissolved in STE buffer. The microconcentrators were previously spin-rinsed with deionized

water to remove traces of glycerol from the membranes, which we found to have a high reactivity with peroxyl radicals. In some experiments either vitamin E or β-carotene was incorporated into the microsomal membrane by sonication as described by Palazzo et al.[36] Samples of whole rat liver cytosol were measured after a final dilution of 1:200, which corresponded to 68 ± 6.3 g protein in the assay (n = 6). The same final dilution was made for both the soluble and proteic fractions. The TOSC values of soluble (deproteinized) and protein fractions were not significantly affected by the different separation procedures. Control experiments confirmed no variation in the partitioning of ethylene between the aqueous and gas phases in biological samples, corn oil, pure antioxidant compounds (e.g., GSH), or organic solvents.

RESULTS

Time dependence of antioxidant protection

Figure 1 shows the time course for antioxidant activity of different concentrations of GSH, ascorbic acid, uric acid, and Trolox. In the presence of the antioxidants, ethylene production from KMBA was quantitatively reduced and higher antioxidant concentrations resulted in longer periods in which ethylene formation was totally inhibited relative to controls.

By plotting the absolute value of the difference between the ethylene peak area obtained at each time point for the sample and control reactions it was possible to visualize if and when the oxyradical scavenging capacity of the solution had become exhausted. This is shown in Fig. 2 for three different concentrations of GSH. At the lowest concentration of GSH (10 μM), its protective effect can be seen to wane as the reaction proceeds; after 108 min the protection afforded by GSH is completely exhausted. In other words, ethylene formation is no longer different from the control (SA-CA = 0) at this point. As the concentration of GSH is increased the oxyradical scavenging capacity is maintained for a correspondingly greater duration as indicated by the suppression of ethylene formation compared to the control throughout the time course studied.

Effect of antioxidant concentration on TOSC

The TOSC of different concentrations of GSH, ascorbic acid, uric acid, Trolox, GSSG, and BSA are reported in Fig. 3. A linear relationship was obtained between TOSC and GSH (10–75 μM), ascorbic acid (5–50 μM), uric acid (1–25 μM), Trolox (2–20 μM), GSSG (0.5–5 mM), and BSA (30–90 μg). From the linear range of TOSC values a specific TOSC may be

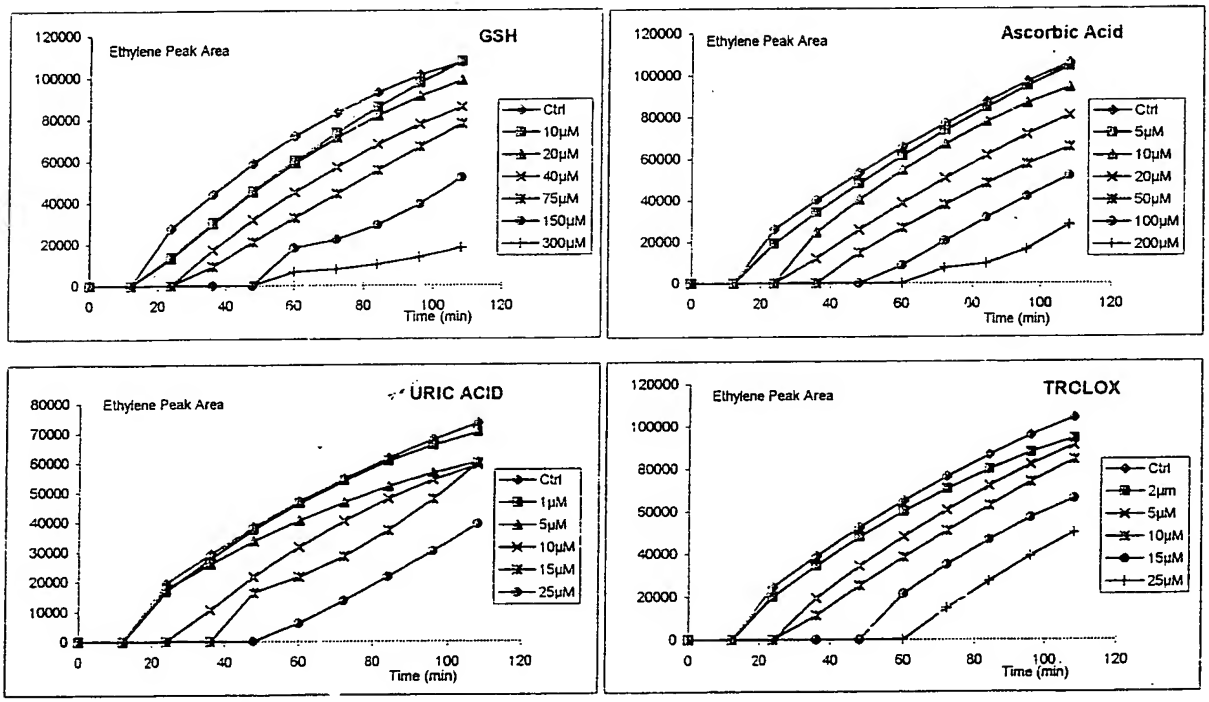

Fig. 1. Time courses of KMBA oxidation by peroxyl radicals in the presence of different concentrations of various antioxidants (reduced glutathione, ascorbic acid, uric acid, Trolox). Data obtained at 35°C.

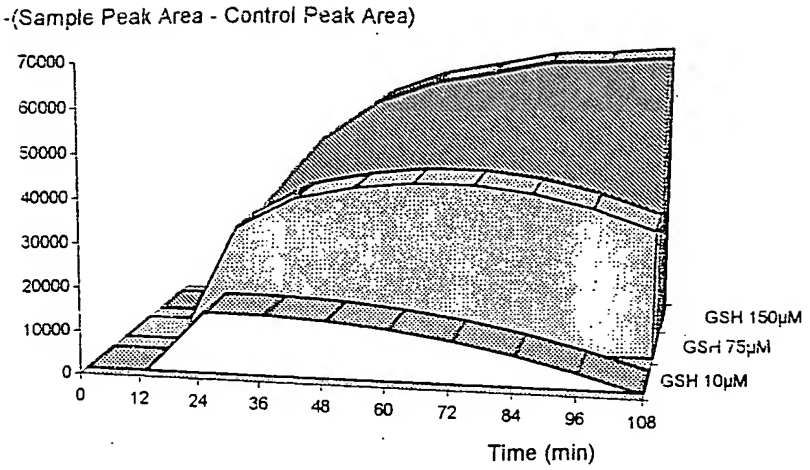

Fig. 2. Variation of the oxyradical scavenging capacity of three concentrations of GSH during the assay. (see Results for explanation). Data obtained at 35°C.

obtained by extrapolation to a final equivalent concentration of 1 μM. Thus, for 1 μM of the antioxidants the specific TOSC values can be given as 0.84 ± 0.10 for GSH, 2.00 ± 0.11 for ascorbic acid, 3.05 ± 0.27 for uric acid, 4.37 ± 0.57 for Trolox, $2.3 \times 10^{-2} \pm 1.0 \times 10^{-2}$ for GSSG, and for 1 μg BSA 0.19 ± 0.02. In this way the marked antioxidant effect of reduced glutathione compared to the oxidized disulfide is readily apparent.

Relative to the primary probe (KMBA in the present case) used to measure the end point (ethylene) of the assay, antioxidants can compete for oxyradicals at varying rates. The ability to distinguish between antioxidants that react faster or slower relative to KMBA or any other probe can be critical in determining their effectiveness during the course of acute and chronic oxyradical influxes. The TOSCA can be used to determine the relative reaction rates of antioxidants (Fig. 4). Fast-acting antioxidants, i.e., Trolox, are those with reaction profiles that indicate a distinct induction period (Fig. 4A) marked by complete inhibition of ethylene production. Antioxidants that react more slowly than KMBA are characterized by reaction profiles without a distinct induction period and which remain linear and show constant inhibition throughout the course of the reaction (Fig. 4B). The latter type of inhibitors have been referred to as "retardants" as opposed to antioxidants[30] to reflect the fact that these compounds react with peroxyl radicals to retard the propagation step of lipid oxidation but do not inhibit it completely. A TOSC value may be calculated for such molecules, albeit an n value may not because n is a function of the induction period of antioxidants.

Effect of antioxidants in combination

The obtained and expected TOSC values for various antioxidants acting alone or in combination are reported in Table 1. When pure solutions of antioxidants were assayed the difference in the obtained TOSC from the expected values ranged between 2 and 8%. In combination, the experimental results were essentially additive with respect to the calculated TOSC values of the individual antioxidants. A slightly higher than additive value for TOSC was obtained when GSH and uric acid were present in combination; the recovered percentage was 127% of the expected sum of the respective single contributions.

Application to rapid screening

The TOSCA can also be used for quick assessment of relative antioxidant potential of samples without having to run the complete time course. By measuring the relative production of ethylene gas at a single time point, one is able to obtain a preliminary measure of the antioxidant activity of a sample. However, this procedure does not enable calculation of TOSC values. By testing several dilutions using this rapid screening technique, relative IC_{50} values could be approximated. Figure 5 illustrates the use of this application for estimating the IC_{50} values of several antioxidants. The order of relative peroxyl radical scavenging was BHA > melatonin = Trolox > uric Acid > ascorbic acid > glutathione, which is consistent with TOSC values for these compounds. Note that this use of the assay does not distinguish between those compounds that completely inhibit KMBA oxidation and those that simply retard it.

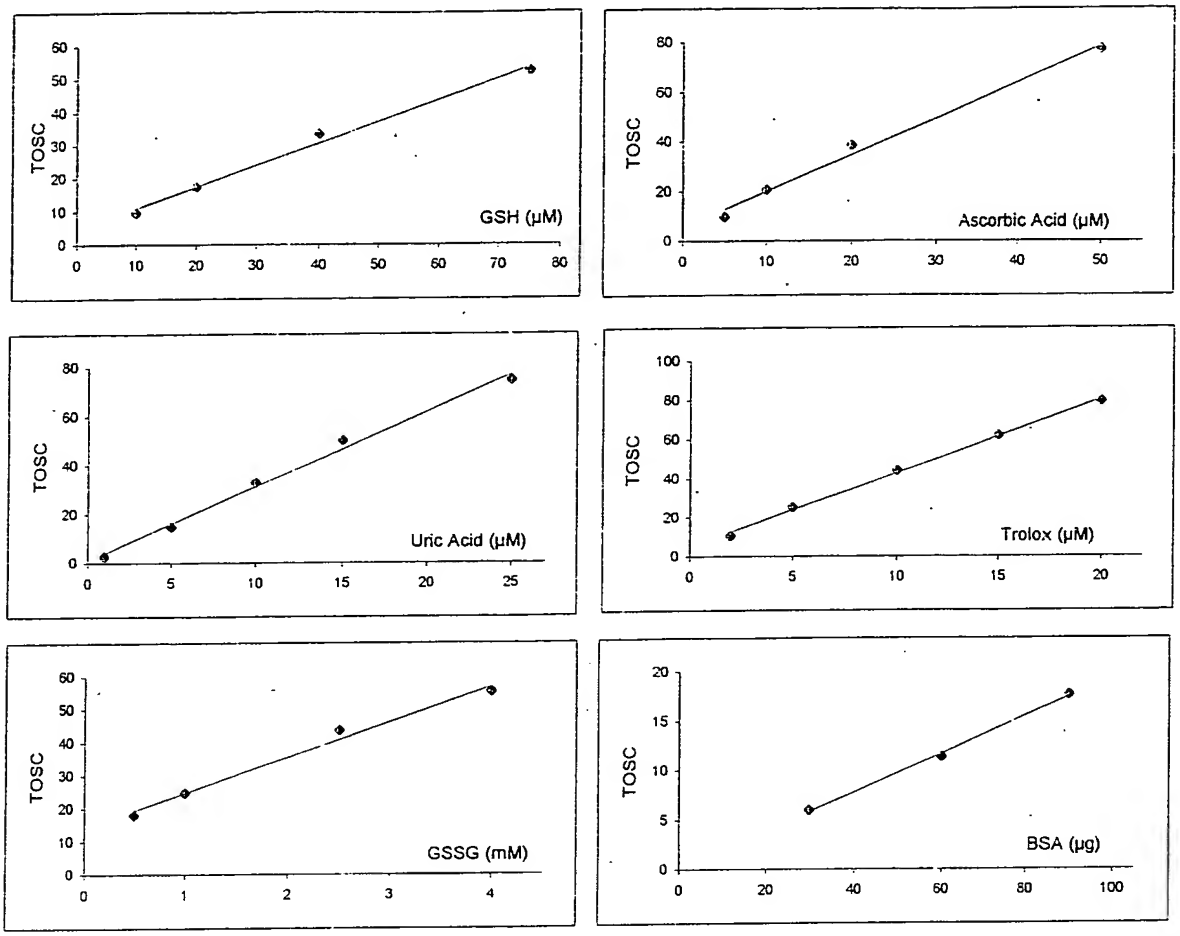

Fig. 3. Regression between TOSC values and different concentrations of antioxidants. GSH: Y = 0.6589 X + 4.3016 (r = .9903); ascorbic acid: Y = 1.4586 X + 5.4469 (r = .99); uric acid: Y = 3.045 X + 0.9379 (r = .9922); Trolox: Y = 3.7711 X + 4.9627 (r = .9974); GSSG: Y = 10.75 X + 13.94 (r = .986); BSA: Y = 0.19X − .007 (r = .998). Data obtained at 25°C.

TOSC of rat liver cytosol

Rat liver cytosol was analyzed as a model biological fluid (Fig. 6). The total oxyradical scavenging capacity (TOSC) of whole cytosol was compared to that of the separate soluble (deproteinized) and proteic fractions (Fig. 6A). It is noted that the soluble fraction accounts for the preponderance of the peroxyl radical scavenging capacity of whole cytosol. Moreover, the TOSC values obtained for these biological fluids were inversely related to their final dilution in the assay (Fig. 6B).

Under our conditions the total oxyradical scavenging capacity (TOSC) of whole cytosol was 27.1 ± 3.6 (n = 6), corresponding to a specific TOSC extrapolated to 1 μg protein of 0.40 ± 0.02. At the same final dilutions used for whole cytosol, the TOSC obtained for the separate soluble and proteic fractions were respectively 20.9 ± 2.2 and 10.4 ± 1.6. The sum of these values was higher (116 ± 8.4%) than those obtained for the corresponding cytosolic fractions and a paired t-test confirmed the statistical significance of these differences ($p < .05$).

The effect of varying the concentrations of both KMBA and ABAP on the TOSC values of whole cytosol is showed in Fig. 7. Raising the concentration of KMBA in the assay above 0.2 mM resulted in an increased competition of KMBA with antioxidants for scavenging peroxyl radicals (Fig. 7A). Figure 7A also shows that the final TOSC values were not significantly altered by varying the duration of the assay. In this respect, a time of 100 ± 5 min from the initiation of the reaction with ABAP allowed sufficient resolution between control and sample reactions while also providing a reasonable number of experimental points for a statistically accurate integration of the areas under the kinetic curves. Lower concentrations of KMBA gave a limited production of ethylene gas. The relationship be-

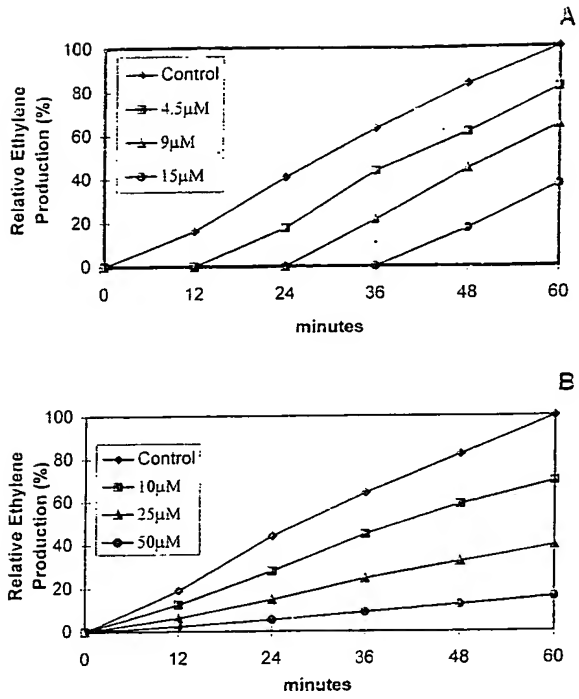

Fig. 4. Time courses of KMBA oxidation by peroxyl radicals in the presence of varying concentrations of (A) Trolox, (B) BHA. Data obtained at 39°C.

tween TOSC and ABAP concentration was fitted by a second-order equation and indicates a maximum TOSC value with 20 mM ABAP (Fig. 7B). For the corresponding microsomal fraction, resuspended in 0.25 M KCl, the TOSC value was 0.15 ± 0.03. The TOSC of microsomes indicated that peroxyl radicals generated in the aqueous phase were scavenged by membrane-associated antioxidants. Moreover, KMBA was a good probe for the scavenging by the microsomal fraction of peroxyl radicals.

Incorporation of vitamin E into the microsomal membrane

To further establish the efficacy of KMBA as a probe for lipid-soluble antioxidant capacity vitamin E was incorporated into the microsomal membrane in essentially as described for β-carotene by Palazzo et al.[36] and compared the ability of these supplemented microsomes to scavenge peroxyl radical with the nonsupplemented microsomes. The results are shown in Fig. 8, where it can be seen that the nonsupplemented microsomes reduce ethylene formation in a dose-dependent manner (TOSC = 0.15 ± 0.03 for 1 μg); thus, the assay is suitable for this subcellular membrane fraction. Vitamin E-supplemented microsomes were significantly better scavengers of peroxyl radical than the nonsupplemented microsomes; thus, the assay can measure the protective effects of lipid-soluble antioxidants. As the concentration of vitamin E was increased in the reaction from 5.3 to 26.8 IU (11.3 to 56.7 mM) concomitantly with microsomal protein concentration so that the vitamin E to protein concentration was kept constant (32 mIU/μg), ethylene production from KMBA was correspondingly decreased, showing that this lipid-soluble antioxidant was protecting KMBA from oxidation by peroxyl radicals. Furthermore, the percentage contribution to antioxidant protection by vitamin E relative to the microsomes themselves is seen to increase with increasing vitamin E even though the ratio of vitamin E per μg microsomal protein remains constant. In other words, at 166 μg protein supplemented with 5.3 IU vitamin E (vit.E/protein ratio =

Table 1. Obtained and Expected TOSC Values for Different Antioxidants Acting Alone or Combined in Mixed Solutions. Expected Values Were Calculated on the Basis of Regression Equations Between TOSC Values and Concentration of Antioxidants

	Obtained TOSC	Expected TOSC	Obtained/Expected %
GSH 20 μM	16.45	17.48	94%
Ascorbic acid 10 μM	18.97	20.03	95%
Uric acid 5 μM	17.50	16.22	108%
GSH 20 μM Ascorbic acid 10 μM	36.64	37.51	98%
GSH 20 μM Uric acid 5 μM	42.86	33.71	127%
Ascorbic acid 10 μM Uric acid 5 μM	34.09	36.25	94%
GSH 20 μM Ascorbic acid 10 μM Uric acid 5 μM	58.04	53.73	108%

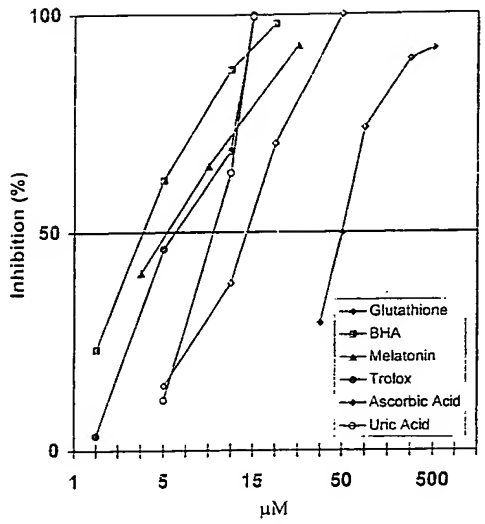

Fig. 5. Percent inhibition vs. concentration of several known antioxidants using the rapid screen method (time = 36 min). Data obtained at 39°C.

32 mIU/μg), the vitamin E inhibited KMBA oxidation 18% more than the microsomes themselves. With 415 μg protein supplemented with 13.3 IU vitamin E, KMBA oxidation was suppressed by about 60% greater than by microsomes alone. At 930 μg protein supplemented with 26.8 IU vitamin E, the supplemented microsomes were 77% more inhibitory than the nonsupplemented microsomes. Similar experiments with β-carotene incorporation indicated this antioxidant to be a poor scavenger of peroxyl radical in our microsomal system. Palozza et al.[36] also found that β-carotene was a poor antioxidant in a rat liver microsomal system with ABAP or the lipid-soluble azo initiator, AMVN.

Determination of the rate of radical input and stoichiometric factors

In assays that measure oxyradical scavenging the rate of radical generation is critical in determining the efficiency of antioxidants. The rate of thermal homolysis for ABAP is concentration and temperature dependent and is defined by equation 3.[37] The value 1.1 $\times 10^{-6}$ is two times the dissociation constant for thermal decomposition of ABAP at 37°C because an ABAP molecule decomposes to generate two radicals.[26,38] Because the rate of radical generation for a given concentration of ABAP can be approximated as linear over the temperature range of 35–39°C (not shown) a function can be calculated from the slope of a temperature vs. rate curve ($M \times s^{-1} \times °C^{-1}$). From this function the rate of radical input for any concentration of ABAP at a specified temperature within the linear range is determined from the y intercept ($M \times s^{-1}$). For 10 and 20 mM ABAP the temperature-dependent rate of radical formation, respectively was calculated as $y = (1.76 \times 10^{-9})x - (5.4 \times 10^{-8})$, $R = 0.9999$ and $y = (3.93 \times 10^{-9})x - (1.3 \times 10^{-7})$, $R = 0.9969$.

$$Rr = 1.1 \times 10^{-6}\,[ABAP]\,s^{-1} \qquad (3)$$

Induction times were used to calculate Rr values in the manner used by Barclay and Ingold.[39] By using the last time point of complete inhibition, a plot of induction times vs. concentration can be constructed. The slope of the regression line for the data gives the relationship shown by equation 4.[40]

$$\text{slope} = \frac{n}{Rr} \qquad (4)$$

where n is equal to the stoichiometric factor (number of radicals scavenged per molecule of antioxidant). Any antioxidant for which the n number is known can be used as a reference antioxidant in the rate calculation. By using Trolox ($n = 2$) as the reference the rate of radical generation by 20 mM ABAP at 35°C is 1.66×10^{-8} M s^{-1}.

Determination of stoichiometric factors for various other antioxidant compounds can also be calculated from the induction time using 2 for Trolox as a standard. The n values for Trolox, ascorbic acid, uric acid, and glutathione are listed in Table 2 along with values reported from other assays for comparison. The trends are very similar for the present and other three methods reported for determining oxyradical scavenging potential.[23,25,29] Uric acid and ascorbate in the TRAP oxygen polarographic-based assay is the only inconsistency. Our values were consistent with both of the phycoerythrin-based assays.

DISCUSSION

We report a simple, reliable gas chromatographic assay for measuring the total oxyradical scavenging capacity (TOSCA) of pure antioxidant solutions or biological fluids. The method is based on the oxidation of KMBA to ethylene upon reaction with certain oxyradicals (equation 1) and on the ability of various antioxidants to inhibit this reaction. The coefficient of variation was 2% ($n = 30$) within a run and 6% ($n = 30$) between runs.

Other methods have been published recently that are similar in concept to the one we present herein. Although there is merit in each of these assays, cer-

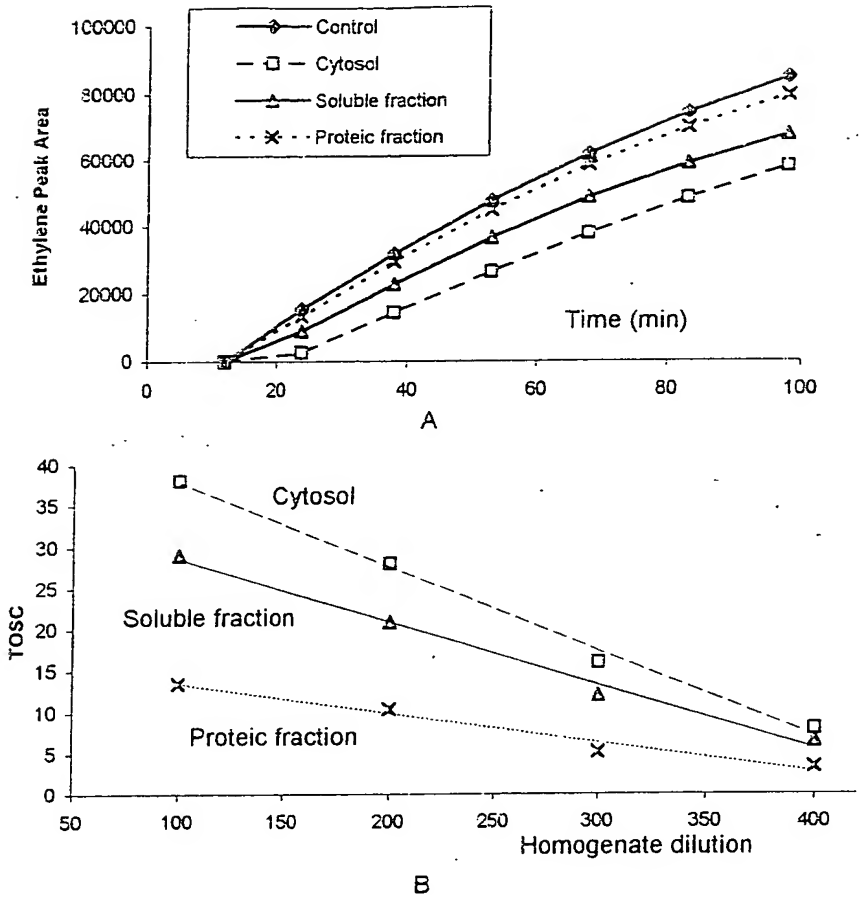

Fig. 6. (A) Time course of relative peroxyl radical scavenging capacity of whole rat liver cytosol, deprotonated cytosol (soluble fraction), and the proteic fraction reconstituted to the original protein concentration of the whole cytosol. (B) The relationship between TOSC values and dilution factor of the whole and fractionated liver cytosol. Data obtained at 35°C.

tain aspects of how they are interpreted have been debated. The method described by Wayner et al.[24] is based on the time necessary to prevent maximum oxygen uptake as an indirect measure of lipid peroxidation by plasma antioxidants. This approach requires a high dilution of the sample to avoid rapid exhaustion of available oxygen; thus, self-termination of lipid peroxidation is competitive with termination afforded by antioxidants.[23,25] By sensitizing the reaction with oxidizable material by Wayner et al.[24] obtained TRAP values (see Introduction) comparable to those measured on neat plasma with a pressure transducer.[39,40] In our method, sample dilution is not so critical; good linearity was obtained between TOSC values and a wide range of final sample concentrations (Fig. 6). Also, the oxidation of KMBA to ethylene (and its quantitative inhibition in the presence of antioxidants) is easily measured without addition of sensitizer.

A fluorescence assay was proposed for rapid screening of molecules with potential ROS-scavenging activity.[26,27] This assay was based on the ability of molecules to inhibit damage to phycoerythrins caused by ROS. The measure of oxyradical-induced damage to phycoerythrins[26,27] depends on the molecular structure of these proteins, which contain 34 tetrapyrrole prosthetic groups, and their absorbance and fluorescence properties, which are highly influenced by the chemical integrity of the protein.[41,42] Because they have a high absorption coefficient and quantum yield,[43] fluorescence analysis of phycoerythrins is sensitive to very low levels (less than 10^{-12} M). Due to the low levels of phycoerythrins used in the PE assay, the decay of fluorescence is too rapid and not linear with respect to peroxyl radicals generated by ABAP at concentrations above 4 mM.[26,28] On the contrary, the sensitivity of the TOSC assay can be enhanced by using higher concentrations of peroxyl radicals. In fact, 20 mM ABAP was found appropriate to increase the control reaction maintaining a low level

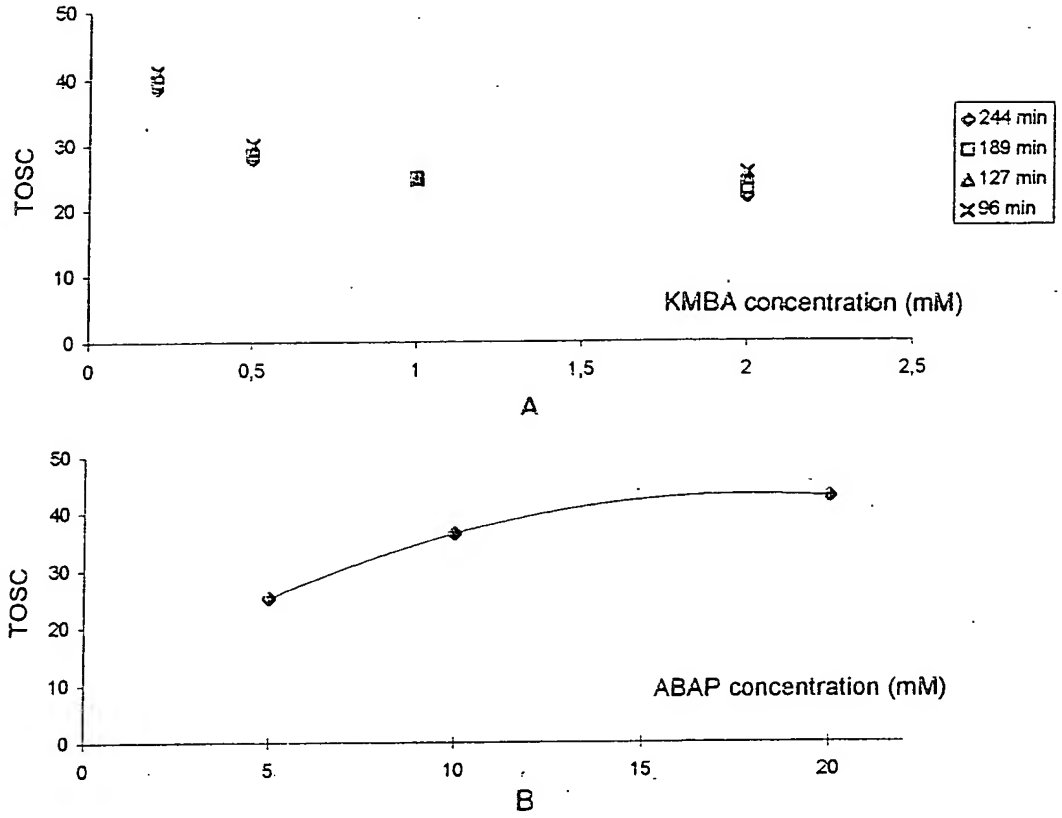

Fig. 7. Effect KMBA and ABAP concentrations on TOSC values of rat liver cytosol with: (A) KMBA varied, ABAP 20 mM; (B) ABAP varied, KMBA 0.2 mM.

of KMBA, 0.2 mM, which at higher doses could compete with antioxidants for peroxyl radicals.

The high sensitivity of the analytical method, together with that for detecting damage to the chemical integrity of phycoerythrins, led to different interpretations in adapting the original assay for quantitative purposes.[28,29,44,45] Based on the different time dependencies of the decrease in phycoerythrin fluorescence in the presence of peroxyl radical scavenging agents, Cao et al.[28] quantified the oxygen radical-absorbing capacity (ORAC) as the net areas under the reaction curves of the control and the sample reactions. In their assay the reaction was driven to completion, i.e., all the soluble antioxidants and most of the proteins were completely oxidized by peroxyl radicals generated from thermal homolysis of ABAP. Concern about his approach was raised by Ghiselli et al.[29] because it is strongly influenced by oxidative damage caused to proteins by peroxyl radicals after the low molecular weight antioxidants are exhausted. Therefore, they proposed quantifying the oxyradical absorbance capacity in terms of the time in which complete protection was afforded by antioxidants (lag-phase) prior to the onset of the rapid decay of phycoerythrin fluorescence. In reply to this concern, Cao and Cutler[45] argued that the linearity of the decrease in phycoerythrin fluorescence emphasized by Ghiselli et al.[29] was not a suitable parameter for use in the quantification of the TRAP assay, because some antioxidants do not afford complete protection, which results in a poorly defined induction period (BHA, for example). On the other hand, Ghiselli et al. argued that the ORAC assay of Cao and Cutler[45] overestimates the true antioxidant potential of a sample by including nonspecific protein interactions. The discrepancies reported by these underscore the difficulty in attempting to define the total antioxidant capacity, especially when biological fluids or mixed solutions are considered.

We are in agreement with Halliwell and Gutteridge[46] that the relative importance of various antioxidants depends on which ROS are under consideration and on their target of damage. As described, the present method, which measures the inhibition of ethylene production from KMBA by antioxidants, does not evaluate the contribution of specific antioxidants in biological fluids to the TOSC. In this respect, correlating the prop-

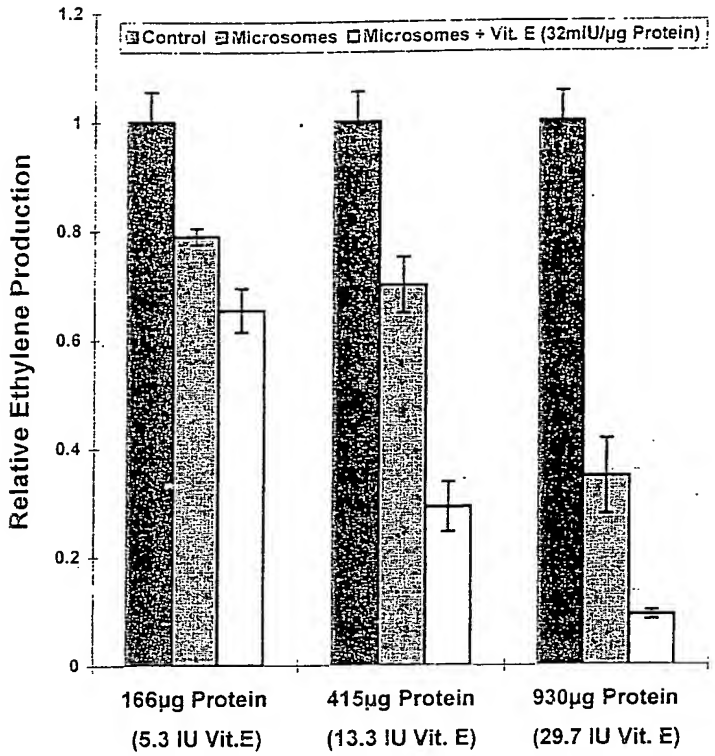

Fig. 8. Inhibition of ethylene production from KMBA in the presence of microsomes and vitamin E (DL-α-tocopherol acetate) supplemented microsomes at 39°C and 10 mM ABAP after a 2-h reaction time. Concentrations of vitamin E/protein are constant at a ratio of 32 mIU/µg of protein.

erties of the anodic waves of low molecular weight antioxidants by cyclic voltammetry to indicate antioxidant status[31] may, by being able to discriminate between different classes of antioxidants, serve as an important adjunct to the methods described herein.

However, an important feature of the KMBA assay is that the quantification of TOSC does not require that the antioxidants be exhausted. This is illustrated in Fig. 2, where it is noted that only the lowest concentration of GSH was completely oxidized at the end of the assay; exhaustion of the total oxyradical scavenging capacity was not observed for higher antioxidant concentrations nor for biological fluids. This approach accounts for the discrepant interpretations by Cao et al. and Ghiselli et al.[28,29,44,45] of the contribution to the radical scavenging capacity after full oxidation of protective agents. Only for protein-containing solutions depleted of soluble antioxidants can the TOSC quantification be partly influenced by a nonspecific reaction of proteins with ROS (see below).

Ethylene is a reasonably reactive molecule and, therefore, the quantum yield of ethylene from KMBA might be altered by the presence of certain compounds. However, any alteration in the yield of ethylene due to such crossreactivity is accounted for in our control reactions, which serves as a "background correction." Unfortunately, when testing biological samples, the control reaction cannot serve as a "background correc-

Table 2. Relative TOSC of Different Antioxidants Calculated from the Present Data and Comparison with Those Derived from the Oxygen Consumption Method[23] and Phycoerythrin Assays[28–29]

	TOSC Present Work	TRAP Assay (oxygen electrode[23])	ORAC (phycoerythrin[28])	TRAP (phycoerythrin[29])
Trolox	1 [2][a]	1 [2]	1 [2]	1 [2]
Uric Acid	0.70 [1.4]	0.65 [1.3]	0.92 [1.84]	0.85 [1.7]
Ascorbic Acid	0.46 [.92]	0.85 [1.7]	0.52 [1.04]	0.75 [1.5]
GSH	0.19 [.38]	0.175 [.35]	—	—

[a] Values in brackets [ ] are the stoichiometric factors discussed in the text.

tion'' owing to the unknown presence of potential ethylene-reactive species. To establish the fate of ethylene in biological samples, we studied the effects of antioxidant addition to biological tissues. We observed that when pure GSH was incubated with biological samples, the TOSC values obtained were additive. Also, when any of our biological samples were incubated in the absence of ABAP, i.e., no peroxyl radical generating species, no ethylene was produced from KMBA indicating that added biological tissues is not a source of radicals in the assay. This data, along with our finding that biological tissues did not alter the partitioning of ethylene between the gas and liquid phases from that of controls, suggests that the fate of ethylene released from KMBA is not altered in the presence of biological tissues. In the presence of an antioxidant that competes for peroxyl radicals with KMBA the formation of ethylene gas is quantitatively reduced. The effects of antioxidants on the rate of KMBA oxidation (ethylene production) are expressed in different ways: (1) the formation of ethylene may be suppresed (retarded) throughout the assay; (2) may be suppressed only for a limited time (if the antioxidant is exhausted); or (3) may be delayed (i.e., a lag-phase occurs). All of these conditions have been obtained using different concentrations of the various antioxidants (Figs. 1 and 4); however, the calculation of TOSC values is not affected by such variations

The oxidation of KMBA to ethylene is about as sensitive to low concentrations of antioxidants as is the method based on the decay of phycoerythrin fluorescence[28]. The specific TOSC for 1 μM solutions were calculated to be 0.84 $\pm$ 0.10 for GSH, 3.0 $\pm$ 0.3 for uric acid, 2.0 $\pm$ 0.1 for ascorbic acid, 4.4 $\pm$ 0.5 for Trolox, and $2.3 \times 10^{-2} \pm 1.0 \times 10^{-2}$ for GSSG. An accurate TOSC value can be calculated only for concentrations of antioxidants within the reported linear ranges. We report a broader linear relationship between TOSC values and GSH (up to 75 μM), uric acid (up to 25 μM), ascorbic acid (up to 50 μM), Trolox (up to 20 μM) and BSA (up to 90 μg) than that of Cao et al.,[28] albeit Cao's approach apparently permits greater precision at the lower ends of the linear ranges. By relating the specific TOSC of various antioxidants to that of Trolox, a relative oxyradical scavenging capacity can be calculated (Table 2) and the results are in good agreement with those derived from other studies.[23,28,29] For soluble antioxidants, the total oxyradical scavenging capacity were of the relative order: Trolox > uric acid > ascorbic acid > GSH.

Synergistic effects between different antioxidants have been suggested,[28,29] but clear evidence of a similar phenomenon is not supported by our data. The TOSC values obtained from our studies of the combined effects of antioxidants in solution, albeit in some circumstances slightly higher than predicted, were essentially additive (Table 1). Additive effects of the antioxidant efficiency of various isolated natural and synthetic antioxidants was also reported by Pryor et al.[30]

When a more rapid, larger scale approach is desired for antioxidant screening, ethylene from KMBA can be measured at a single time point; this permits the number of different samples or the range of concentrations to be varied. When used in this manner, the results are of a more qualitative nature, i.e., the differences in the rates of reaction of the antioxidants with the peroxyl radical are not accounted for. This technique is suitable for preliminary evaluation of suspected antioxidants or to approximate concentration ranges to be tested for TOSC calculations.

When biological fluids were analyzed, the TOSC was linear with dilution of whole cytosol, soluble, and protein fractions, and oxyradical scavenging capacity was not exhausted during the assays (Fig. 6). The TOSC values can be easily related to traditional parameters (i.e., per μg protein or mg tissue) allowing further comparisons between the oxyradical scavenging capacity of different tissues and/or organisms.

The TOSC value for whole cytosol of rat liver was 0.40 $\pm$ 0.02 per μg protein. When whole cytosol was separated into soluble and protein fractions, the sum of their separate TOSC values was consistently greater than that obtained for the corresponding whole cytosol from which they were derived. During analysis of whole cytosol endogenous antioxidants are not exhausted, indicating that fast-reacting molecules scavenge peroxyl radicals at a much higher rate than proteins.[27] This minimizes random damage to proteins on the TOSC value. For the pure protein fraction the obtained TOSC is likely to be a combination of both thiol content and random damage. If we consider the value of TOSC for BSA to be a representative value of the random damage of generic protein content, then random damage to the rat liver protein fraction would account for about 60% of its TOSC. For whole cytosol and soluble antioxidants TOSC reflect only specific oxyradical scavenging capacity; thus, the rat liver soluble fraction accounted for 77.2 $\pm$ 6.8% of the protective antioxidant potential of the whole cytosol.

The KMBA assay was effective in detecting the antioxidant capacity of lipid- or oil-soluble antioxidants. Microsomal membranes and vitamin E-supplemented membranes exerted substantial antioxidant capacity toward peroxyl radicals even when they were generated in aqueous solution. Barclay et al.[47] showed that ABAP partitions about 91% into the micellar phase of SDS micelles, a distribution very similar to that with linoleic acid (95%). Therefore, the effectiveness of the KMBA

assay toward lipid-soluble antioxidants is consistent with the suggestion of Pryor et al.[30] that most of the primary radicals from ABAP are generated within or at the surface of the membrane micellar system. Thus, lipid-soluble scavengers should behave quite normally in the assessment of TOSC by biological tissues.

Ghiselli et al.[29] reported a rapid loss of TRAP measured with the phycoerythrin assay for samples maintained at −80°C for more than 72 h. We found that if samples are aliquoted, stored at −80°C, and thawed only once, the TOSC values were stable for at least 6 weeks, as reported also by Chevion et al.[31] using a cyclic voltammetry assay.

In conclusion, the TOSC assay is a reliable method for antioxidant solutions and biological tissues. It gives linear responses over a wide range of final dilutions of biological fluids. The ease of the analytical procedure, high reproducibility of the results, and the ability to analyze 8–10 samples in less than 2 h (20–30 samples if a single time period is used) make this a useful procedure for measurements of the total oxyradical scavenging capacity and its relationship with oxidative stress resistance. We emphasize that our gas chromatographic technique is not suggested to supplant other assays in the literature designed for similar purposes, but to extend the technology of assessing and quantifying oxyradical scavenging capacity of antioxidants to a wider range of investigators. We have presented our assay in terms of its peroxyl radical scavenging (absorbance) capacity as this radical is the basis of the other assays cited to which we compare the present. We name our assay total oxyradical scavenging capacity (TOSC) to reflect its utility in quantifying not only peroxyl radical scavenging but also other radicals (e.g., hydroxyl, trichloromethyl, alkoxyl, and alkyl) and nonradical oxidants that react with KMBA to produce ethylene, for example, HOCl[34] and peroxynitrite.[35] As described, the TOSC assay is not effected by endogenous levels of these nonradical oxidants in biological tissues because their levels are negligible compared to the amount of peroxyl radical generated in the reaction from ABAP. Characterization of the interaction of KMBA with this spectrum of radicals is currently under study in our laboratory.

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ABBREVIATIONS

ABAP—2–2′-azobis(2-amidinopropane)
AMVN—2,2′-azobis(2,4-dimethylvaleronitrile)
BSA—bovine serum albumin
GSH—reduced glutathione
GSSG—oxidized glutathione
KMBA—α-keto-γ-methiolbutyric acid
ORAC—oxygen-radical absorbance capacity
PE—phycoerythrin
TOSCA—total oxyradical scavenging capacity assay
TOSC—total oxyradical scavesnging capacity
TRAP—total radical-trapping antioxidant parameter
TROLOX—6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid